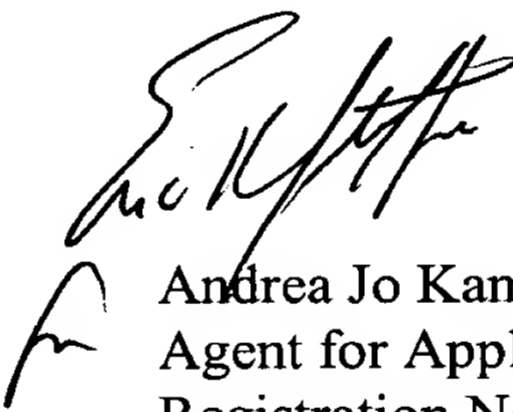## *Remarks*

Applicant submits herewith Hickman *et al.*, *Journal of Immunology*, *171*: 22-26 (2003) (Exhibit A) and Herberts *et al.*, *Human Immunology*, *64*:44-55 (2003) (Exhibit B). These references are submitted to supplement the Reply filed on October 15, 2003.

Applicant respectfully requests that the Examiner consider the references in conjunction with the October 15, 2003 Reply. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Consideration of this Supplemental Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

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(side text) Polyclonal Anti-Immunoglobulin

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Cutting Edge: Class I Presentation of Host Peptides Following HIV Infection[1]

Heather D. Hickman, * *Angela D. Luis,* * *Wilfried Bardet,* * *Rico Buchli,* * *Casey L. Battson,[†] Michael H. Shearer,[2]* Kenneth W. Jackson,[†] Ronald C. Kennedy,[2]* and William H. Hildebrand[3]* *

Class I MHC molecules bind intracellular peptides for presentation to cytotoxic T lymphocytes. Identification of peptides presented by class I molecules during infection is therefore a priority for detecting and targeting intracellular pathogens. To understand which host-encoded peptides distinguish HIV-infected cells, we have developed a mass spectrometric approach to characterize HLA-B*0702 peptides unique to or up-regulated on infected T cells. In this study, we identify 15 host proteins that are differentially presented on infected human T cells. Peptides with increased expression on HIV-infected cells were derived from multiple categories of cellular proteins including RNA binding proteins and cell cycle regulatory proteins. Therefore, comprehensive analysis of the B*0702 peptide repertoire demonstrates that marked differences in host protein presentation occur after HIV infection. The Journal of Immunology, 2003, 171: 22–26.

M ajor histocompatibility complex class I molecules exist as heterotrimers composed of a H chain, L chain, and peptide ligand (1). Class I molecules sample peptides from the proteome of the cell, transport the peptides to the surface, and interface with immune effectors where they communicate cellular fitness (2). This ability to sample and report on vast numbers of intracellular proteins has earned class I molecules the nickname "nature's gene chips" (3).

Although it has been demonstrated that MHC molecules sample a vast array of endogenous proteins during the normal cellular lifecycle, characterization of host-protein-derived peptides after HIV infection has not been performed. Therefore, a fundamental question arises: what host-encoded peptides are uniquely presented on the surface of infected cells? Based upon the observation that HIV produces and interacts with multiple host-encoded proteins inside the cell (including Tsg101 (4) and

RNA polymerase II (5)), we hypothesized that host-protein-derived peptides are uniquely presented during infection.

We previously described a bioreactor-HLA-protein production method and a mass-spectrometric-ion-mapping system for comparatively screening class I-eluted peptide ligands (6, 7). In this study, we extend this approach to test the hypothesis that HIV infection alters the presentation of host-encoded peptides. Peptides eluted from HLA-B*0702 molecules produced in HIV-infected or uninfected cells were directly compared using mass spectrometry. Comparative mapping of HIV-infected and uninfected peptides results in the identification of 15 host-derived peptides uniquely presented on HIV-infected cells.

Materials and Methods
Soluble HLA production

Soluble HLA-B*0702 transfectants were produced as described (6) using Sup-T1 cells (7). Transfectants were cultured in a Unisyn CP2500 bioreactor unit (Biovest International, Minneapolis, MN) for 2 mo with continuous peptide collection. Approximately 30 mg of soluble HLA (sHLA)[4] were collected from either uninfected or infected cells, supplemented with 1% Triton X-100, and stored at 4°C.

HIV infection

HIV-1 strain MN was propagated in Sup-T1 transfectants and monitored by p24 ELISA (Zeptometrix, Buffalo, NY). For cell pharm infection, 3 × 10⁹ cells were infected at a multiplicity of infection (MOI) of 0.5. For time course protein analysis, 1 × 10⁹ cells were infected at an MOI of 4.5 for 2 h, washed once, and replaced in RPMI 1640 + 20% FBS.

Peptide purification

B*0702 molecules were affinity purified over a W6/32 affinity column. Peptides were eluted with 0.2 N acetic acid, brought to 10% acetic acid concentration, and heated to 78°C for 10 min. Fractions were purified in a stirred cell with a 3-kDa molecular mass cutoff cellulose membrane (Millipore, Bedford, MA). Peptides were reversed-phase-HPLC fractionated using a standard gradient of acetonitrile. Separate but identical peptide purifications were done from uninfected and infected cells.

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[4] Abbreviations used in this paper: sHLA, soluble HLA; MOI, multiplicity of infection; PARP, poly(ADP-ribose) polymerase; MS, mass spectroscopy; HMG, high-mobility group protein; eIF, eukaryotic translation initiation factor; USP3, ubiquitin-specific protease 3; E6BP, E6-binding protein; HSP27, heat shock protein 27; TCP, tailless-complex protein; PTB, polypyrimidine tract-binding protein; Hu R, Hu Ag R.
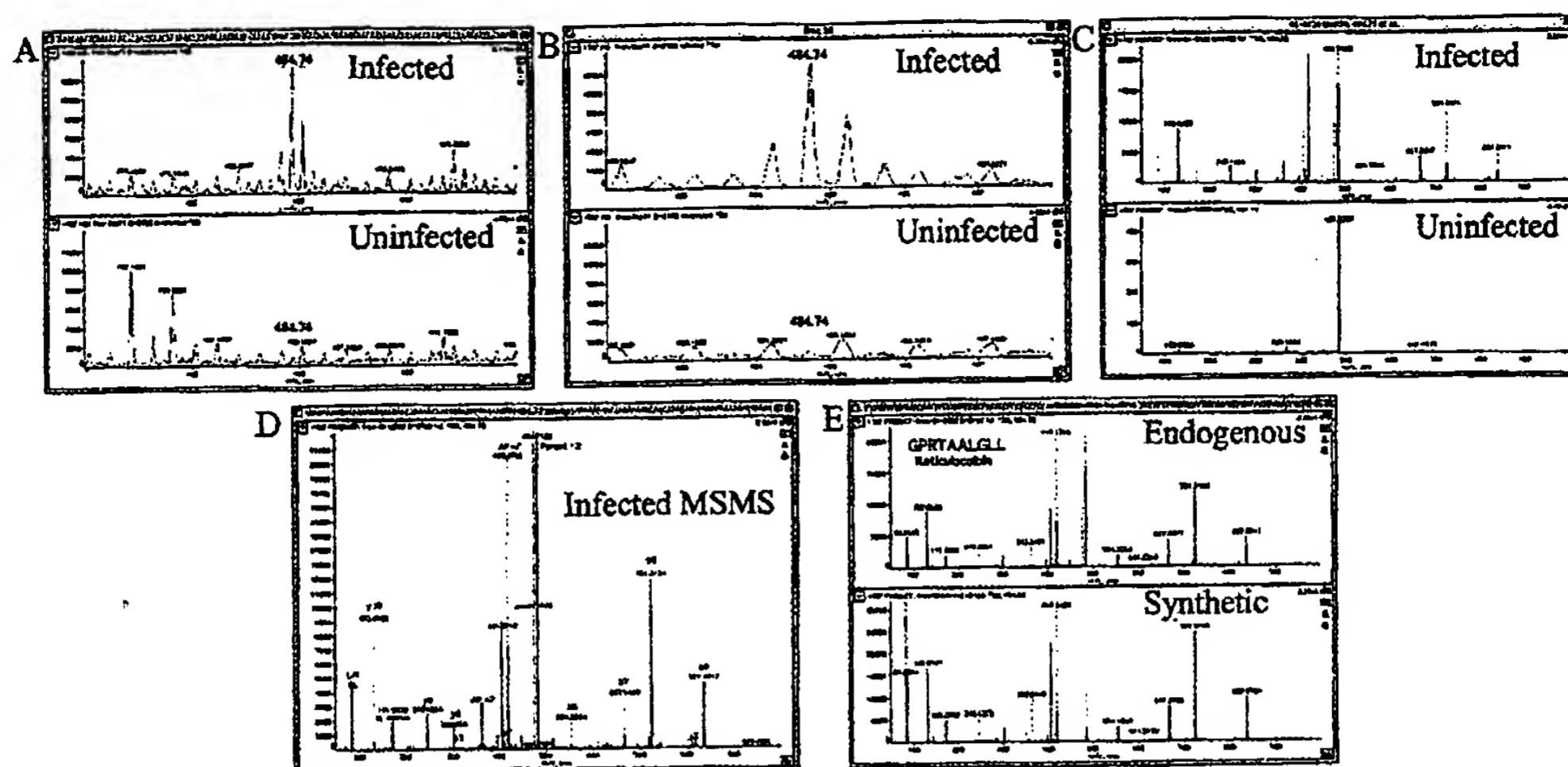
FIGURE 1. Mass spectrometric analysis of HLA-B*0702-bound peptides in HIV-infected cells. Peptides eluted from affinity purified sHLA were mapped on an ESI-QTOF mass spectrometer. Spectra were scanned for ion differences unique to infected cells. *A*, Initial scans were performed with windows of 200 atomic mass units and (*B*) subsequently zoomed to identify ion differences. *C*, Identified differences were subjected to MS/MS. *Top panel*, Peptide from uninfected cells; *bottom panel*, fragmentation of identical ion with identical atomic mass units in the uninfected fraction. Different fragmentation patterns indicated the absence of the peptide in the uninfected fraction. *D*, MS/MS sequence assignment from differentially expressed peptide. *E*, Synthetic peptides corresponding to putative sequence were subjected to MS/MS to verify sequence integrity.

Mass spectrometric analysis

Fractionated peptides were mapped by mass spectrometry as described (7). Peptides were nanoelectrosprayed (Protana, Odense, Denmark) into a Q-Star QTOF mass spectrometer (PerSeptive Sciex, Foster City, CA). Spectra from the same fraction in uninfected/infected cells were aligned to the same mass range and visually assessed for the presence of differences that were selected for manual and automated sequence assignment using the programs BioMultiview (PerSeptive Sciex) and MASCOT (Matrix Science, London, U.K.) (8). Synthetic peptides corresponding to each putative sequence were produced and subjected to mass spectroscopy MS/MS under identical collision conditions as the naturally occurring peptide and overlaid to confirm sequence identity.

Peptide binding assay

IC$_{50}$ values were determined using the HLA-B*0702 PolyScreen kit (Pure Protein, Oklahoma City, OK) according to the manufacturer's instructions. Fluorescently labeled control peptide and sHLA were incubated with each test peptide until equilibration of peptide replacement was reached as read on an Analyst AD plate reader (Molecular Devices, Sunnyvale, CA). IC$_{50}$ values were calculated using a dose-response curve.

Real-time PCR and Western blots

For Western blots, cells were lysed in electrophoresis buffer and total protein was quantified using the BCA Protein Analysis kit (Pierce, Rockford, IL). Proteins were transferred onto nitrocellulose membranes (Osmonics, Westborough, MA) before probing and detecting with commercially available Abs. For real-time PCR, total RNA was isolated using the Total RNA Isolation kit (Ambion, Austin, TX) and cDNA synthesized using the Retroscript kit. PCR was performed on a PE-7700 Light Cycler (Applied Biosystems, Foster City, CA) using primer pairs designed using Primer Express software. β-Actin was used as an internal standard. Relative transcript levels were calculated using the δ-δ cycle threshold method and normalized to zero in the uninfected cells.

Apoptosis analysis

Infected cells were treated with the Apo-Direct kit (BD Biosciences, Mountain View, CA) according to manufacturer's instructions and followed by analysis on a FACSCalibur. Poly(ADP-ribose) polymerase (PARP) cleavage was detected from the same Western blot lysates as above with an anti-PARP Ab (BD Biosciences).

Results and Discussion

Mass spectrometric mapping of peptides from infected and uninfected cells identifies 15 unique host-derived peptides

Following the harvest of B*0702/peptide complexes from both infected and uninfected cells, peptide ligands were eluted, fractionated, and each fraction was comparatively mapped using MS. Multiple peptides unique to HIV-infected cells were identified in these MS spectra (Fig. 1*A*). For example, comparison of spectra produced with peptides eluted from infected and uninfected cells identified a peak unique to the infected cells at 484.7 atomic mass units of fraction 16 (Fig. 1*B*). Peptide peaks unique to or up-regulated on HIV-infected cells were analyzed

Table I. *IC$_{50}$ values for peptide binding to sHLA-B*0702*

Peptide Sequence	IC$_{50}$ (nM)
TPQDLNTML[a]	3047
SPRTLNAWV[a]	254.8
AASKERSGVSL[b]	317.3
AARPATSTL	209.8
APAYSRAL	214.7
APKRPPSAF	176.1
GPRTAALGLL	257.9
IATVDSYVI	218,900
IPCLLISFL	5,980
LPQANRDTL	598.7
LPTSHPKIV	3,563
NPNQNKNVAL	1,581
QPRYPVNSV	945.5
RPYSNVSNL	227.5
SPNQARAQAAL	926.3
STTAICATGL	248,100
TPQSNRPVm	466

[a] Control peptide, HIV-derived.
[b] Control peptide, self-derived.

Table II.   *HLA-B*0702 peptides differentially presented on infected cells*

Peptide Sequence	Source Protein	Abbreviation	Main Cellular Function
APKRPPSAF	High mobility group protein 1/2	HMG 1/2	DNA binding
TPQSNRPVm[a]	RNA polymerase II, polypeptide A	RNA pol	Transcription
SPNQARAQAAL	Polypyrimidine tract-binding protein	PTB	mRNA processing and stability
NPNQNKNVAL	Hu Ag R (ELAV-like 1)[b]	Hu R	mRNA processing and stability
AARPATSTL	Eukaryotic translation initiation factor 4GI	eIF4G	Translation
QPRYPVNSV	Tailless complex protein 1, α polypeptide	TCP-1	Cytoplasmic protein chaperone
APAYSRAL	Heat shock protein 27	HSP27	Cytoplasmic protein chaperone
STTAICATGL	Ubiquitin-specific protease 3	USP3	Ubiquitin pathway
GPRTAALGLL	E6 binding protein (reticulocalbin 2)[b]	E6BP	Unknown; ubiquitin pathway?
IPCLLISFL	Cholinergic receptor, α 3 polypeptide	CholR	Signal transduction
RPYSNVSNL	Set-binding factor 1	SBF-1	Cell growth and differentiation
LPQANRDTL	MgcRacGap	MRG	Cell growth and differentiation
LPTSHPKIV	Suppressin	Sup	Cell cycle regulator
MAMMAALMA	Spark-like protein 1 (hevin)[b]	Spark-like	Antiadhesive extracellular matrix protein
IATVDSYVI	Tenascin C (hexabrachion)[b]	TenC	Similar to antiadhesive extracellular matrix protein

[a] Oxidized methionine.
[b] Protein possessing more than one common name.

with tandem MS (Fig. 1, *C* and *E*). De novo sequence identification from MS/MS fragmentation patterns was performed on each peak unique to or up-regulated on infected cells (Fig. 1, *D* and *E*). Putatively identified ligands were analyzed for their predicted tandem MS fragmentation pattern (Fig. 1*D*) and compared with the spectra produced from uninfected cells (Fig. 1*C*). As a final confirmation of sequence integrity, peptides corresponding to the putative ligand sequences were synthesized and fragmented (Fig. 1*E*). Only peptides with identical experimental and control MS/MS fragmentation patterns were selected for further analyses.

As an independent verification of correct sequence assignment, we performed peptide-binding assays on each of the peptides identified through mass spectrometry. Synthetic peptides representing the peptide sequence were used in competition against a fluorescently labeled control peptide for binding to sHLA B*0702 molecules; IC_{50} values were established for each peptide (Table I). All synthetic peptides bound to HLA-B*0702, most as strongly as the B*0702 control peptides. These data, combined with MS/MS fragmentation pattern analysis, confirmed that MS sequence analysis had yielded the correct ligand sequences.

Peptides presented at altered levels during HIV infection were derived from host proteins involved in multiple cellular pathways

Comparison of mass spectra from peptides eluted from infected and uninfected cells yielded 15 self protein-derived peptides showing altered expression on HIV-infected cells. These host-encoded peptides could be categorized by primary cellular function (Table II). Several of the peptides derived from proteins involved in RNA transcription or translation; peptides NPN-QNKNVAL and SPNQARAQAAL are both fragments of mRNA binding proteins, while the source protein for AP-KRPPSAF, high-mobility group protein (HMG) 1, facilitates the binding of transcription factors to DNA sequences (9). Another ligand in this category (AARPATSTL) derived from eukaryotic translation initiation factor (eIF) 4GI, a protein that plays a key role in cap-dependent mRNA translation initiation (10).

A second category of peptides unique to HIV-infected cells derived from cellular proteins involved in protein regulation. Peptides STTAICATGL and GPRTAALGLL derived from

ubiquitin-specific protease 3 (USP3) and E6-binding protein (E6BP), respectively. Protein USP3 hydrolyzes ubiquitin-protein bonds while E6BP can bind and alter the activity of a known ubiquitin ligase (11). Peptides APAYSRAL and QPRY-PVNSV derived from source proteins heat shock protein 27 (HSP27) and tailless-complex protein (TCP)-1 (Table II) and participate in protein folding as cytoplasmic chaperones (12, 13).
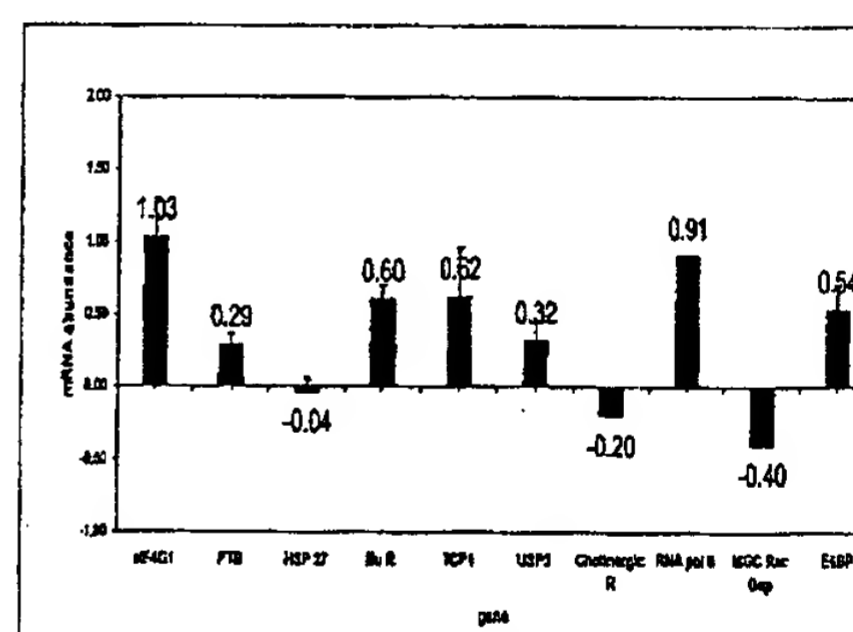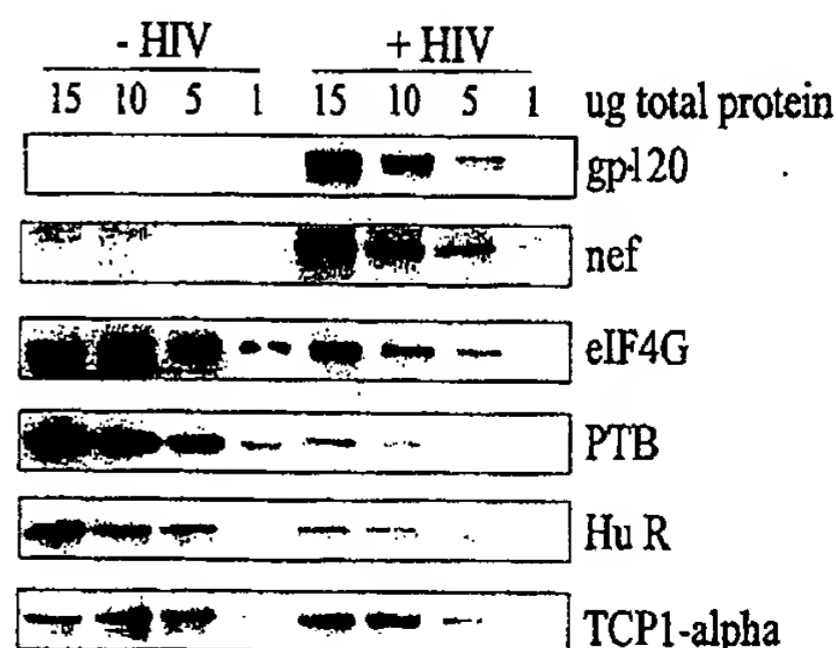

FIGURE 2.   Transcriptional and protein levels of peptide-source proteins during a mixed-HIV infection of sHLA-B*0702 transfectants. *A*, Relative transcript levels of peptide source proteins as determined by real-time PCR. *B*, Western blot analysis of peptide-source proteins before (−HIV) and after (+HIV) infection.
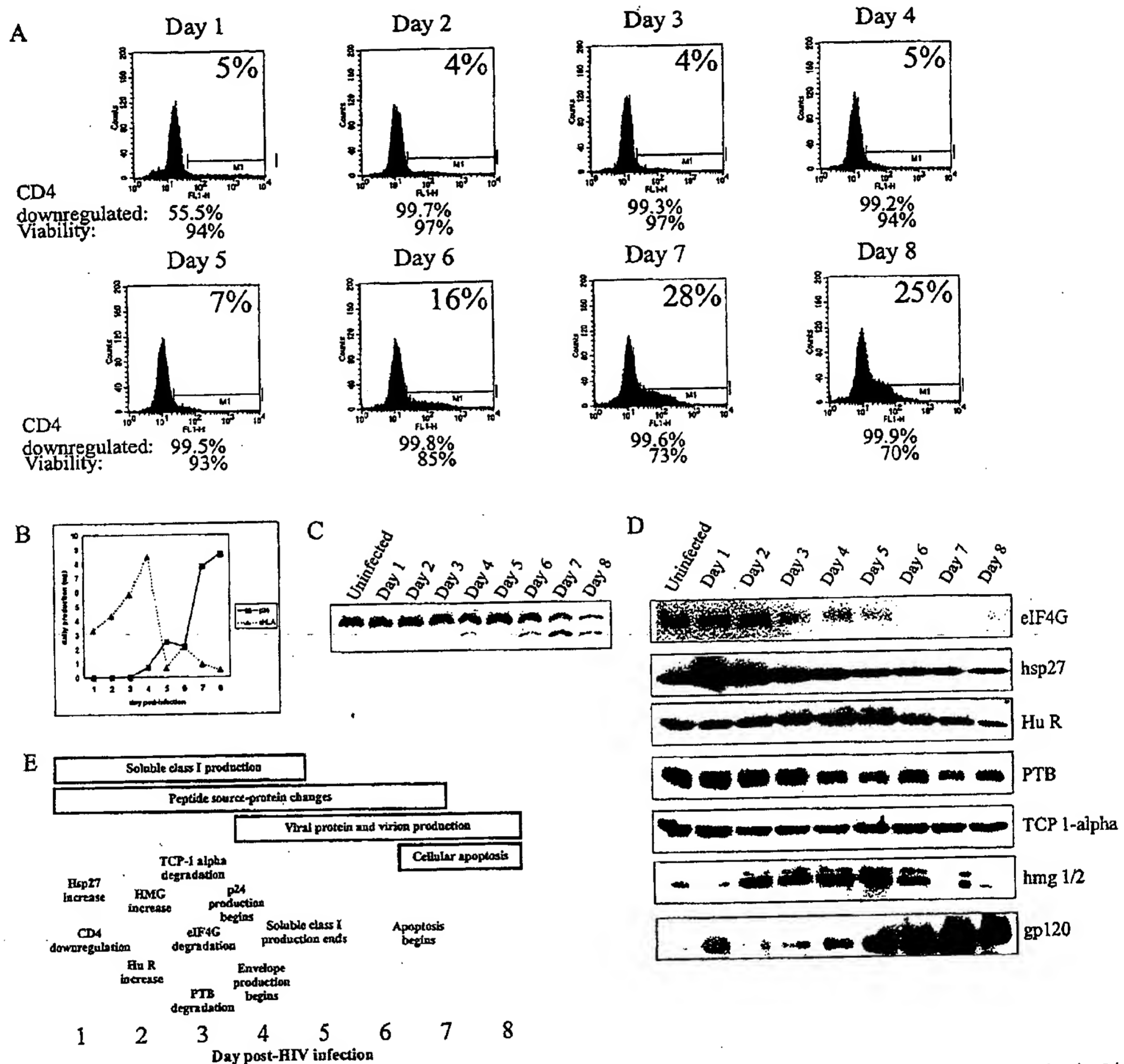
FIGURE 3. Kinetics of protein alterations during HIV infection of sHLA-B*0702 transfectants. Transfectants were infected with HIV MN and analyzed at 24-h intervals postinfection. *A,* Percentage of cells undergoing apoptosis and CD4 down-regulation during infection as determined by flow cytometry. Percent viability was determined by forward and side scatter. *B,* sHLA secretion and p24 secretion as measured by ELISA. *C,* Apoptosis analysis by Western blotting for PARP cleavage. *D,* Western blots of proteins representing unique peptides. *E,* Kinetics of protein changes during infection.

Unique peptides were derived from host proteins with altered protein levels during infection

Unique presentation of peptides during infection could be the result of multiple alterations inside the infected cell. Because HIV has been shown to transcriptionally up-regulate cellular genes (14) and gene overexpression can result in presentation by class I molecules (15), transcriptional up-regulation of genes representing unique peptides could be occurring before peptide presentation. To test this interpretation, real-time PCR was performed on mRNA transcripts from infected cells (Fig. 2*A*). Because the bioreactor system used for the harvest of sHLA proteins from infected cells represented a mixture of cells at different points in infection, T cells were infected with HIV and fed biweekly with uninfected cells. Once syncitia were evident visually, cells were pelleted, lysed, and real-time PCR was performed on ex-

tracted mRNA. Little change in the transcriptional level of the proteins examined was found. Thus, there was no pattern of transcriptional activation that would account for an overabundance of particular MHC-bound peptides on the infected cells.

A second possible mechanism for the presentation of unique peptides during infection was fluctuation in the levels of cellular proteins from which the peptides were derived. To examine this possibility, we performed Western blotting on total protein lysates from the same infected cells examined by real-time PCR. All of the proteins examined—eIF4GI, polypyrimidine tract-binding protein (PTB), Hu Ag R (Hu R), TCP-1, and HSP27—decreased in protein level in infected cells, indicating that degradation or turnover of the proteins was occurring (Fig. 2*B*). Thus, protein degradation during infection was the general mechanism for unique peptide presentation during infection.

Peptide changes occur early in infection

To determine the timing of protein changes, we performed a time course infection with HIV. Sup-T1 T cells were infected with HIV-1 MN at an MOI of 4.5 and cells and supernatants were sampled at 24-h intervals postinfection. Primary indicators of HIV infection were apparent early in the time course; almost 100% of cells exhibited down-regulation of CD4 by day 2 (Fig. 3A), while p24 release from infected cells began on day 4 (Fig. 3B). Flow cytometric measurement of cell viability and apoptosis (Fig. 3A) showed that a majority of the cells remained viable throughout the infection while TUNEL staining indicated that only one-fourth of the cells were undergoing apoptosis by day 8. As a secondary measurement of apoptosis, PARP cleavage was detected on day 7 (Fig. 3C). Interestingly, secretion of class I from infected cells precipitously dropped at day 5 (Fig. 3B), before the onset of apoptosis. These data indicate that cell death and apoptosis occur at late time points during infection of Sup-T1 T cells with HIV strain MN and that class I secretion predominates at early time points.

After establishing the kinetics of infection, Western blots were performed on the host proteins represented by overabundant peptides (Fig. 3D). Three of the proteins exhibited degradation by day 3 postinfection, including eIF4G, TCP-1α, and PTB. Western blots further showed that HSP27, Hu R, and HMG 1/2 were up-regulated 1–2 days postinfection before dropping to lower levels later in the infection. The viral proteins envelope (gp120) and p24 (by ELISA) became apparent at day 4 of infection. As demonstrated in the mixed bioreactor infection, decreased levels of source proteins eIF4G, TCP-1 α, and PTB during HIV infection were not a result of decreased mRNA production (measured by real-time PCR at days 1–4, data not shown). Taken together, these data indicate that HLA-B*0702 presentation of unique peptides occurs as a result of protein changes occurring early in HIV infection (Fig. 3E).

In summary, the analysis of the HLA-B*0702 repertoire after HIV infection reveals a series of host-protein-derived peptides presented uniquely by infected cells. The host peptides most likely are presented as the result of protein level fluctuations that occur early during HIV infection. The consequence of these overabundant self peptides during infection is currently unknown but fits well into the paradigm of autoimmunity; autoimmune reactions are often present in individuals suffering from AIDS (16). Immune recognition of virus-induced host epitopes such as those reported in this study could function in the induction of autoimmune responses directly through increasing the concentration of self peptides on the cell surface.

Indeed, this mechanism is supported by a recent study demonstrating autoreactivity following measles virus-induced up-regulation of self peptides (17). Irrespective of possible function, these host-derived peptide ligands provide an expanded view of peptide presentation to the immune system following viral infection.

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Human Immunology

Selected Papers to Appear in Forthcoming Issues

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STUCK, PANI, BESROUR, SEGNI, KRAUSE, USADEL, and BADENHOOP
  Fas Ligand Gene Polymorphisms Are Not Associated With Hashimoto's Thyroiditis and Graves' Disease

Autoreactivity Against Induced or Upregulated Abundant Self-Peptides in HLA-A*0201 Following Measles Virus Infection

Carla A. Herberts,
Jacqueline van Gaans-van den Brink,
Ed van der Heeft, Margot van Wijk, Jan Hoekman,
Assan Jaye, Martien C. M. Poelen, Claire J. P. Boog,
Paul J. M. Roholl, Hilton Whittle, Ad P. J. M. de Jong,
and Cécile A. C. M. van Els

ABSTRACT: Infectious agents have been implied as causative environmental factors in the development of autoimmunity. However, the exact nature of their involvement remains unknown. We describe a possible mechanism for the activation of autoreactive T cells induced by measles virus (MV) infection. The display of HLA-A*0201 associated peptides obtained from MV infected cells was compared with that from uninfected cells by mass spectrometry. We identified two abundant self-peptides, $IFI-6-16_{74-82}$ and $Hsp90\beta_{570-578}$, that were induced or upregulated, respectively, following infection. Their parental proteins, the type I interferon inducible protein IFI-6-16, and the β chain of heat shock protein 90, have not been involved in MV pathogenesis. MV infection caused minor and major changes in the intracellular expression patterns of these proteins, possibly leading to altered peptide processing. $CD8^+$ T cells capable of recognizing the self-peptides in the context of HLA-A*0201 were detectable at low basal levels in the neonatal and adult human T cell repertoire, but were functionally silent. In contrast, peptide-specific producing IFN-γ producing effector cells were present in MV patients during acute infection. Thus, MV infection induces an enhanced display of self-peptides in MHC class I, which may lead to the temporary activation of autoreactive T cells. *Human Immunology 64, 44–55 (2003).* © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Science Inc.

KEYWORDS: measles virus; autoimmunity; MHC class I epitopes

ABBREVIATIONS

B-LCL	B-lymphoblastoid cell line
CAD	collision activated dissociation
CBMC	cord blood mononuclear cells
ELISPOT	enzyme-linked immunospot assay
HIV-1	human immunodeficiency virus-1
HPLC	high performance liquid chromatography
Hsp90β	heat shock protein 90 β chain
IFI-6-16	type I interferon inducible protein
IFNγ	interferon γ
μLC-ESI-MS	microcapillary rpHPLC electrospray ionisation mass spectrometry
MS	mass spectrometry
MV	measles virus
MV-H	measles virus hemagglutinin
m/z	mass to charge ratio
PBMC	peripheral blood mononuclear cells
rpHPLC	reversed phase high performance liquid chromatography
SFC	spot forming cell
SPA	N-hydroxysuccinimidyl-3-pyridylacetate

INTRODUCTION

Potentially autoreactive T cells that have escaped negative selection in the thymus are present in the periphery, but they are harmless provided they are not activated and their specific self antigen remains unexpressed on professional antigen-presenting cells (APC) in lymphoid organs [1, 2]. Despite the various mechanisms that operate to ensure peripheral tolerance [3], autoreactive T cells can be activated *in vivo* by viral or bacterial infections [4, 5], which may lead to the induction of autoimmune diseases. Many clinical findings have correlated autoimmune conditions with a particular infectious agent [6]. Measles virus (MV) infection has been associated with several autoimmune diseases [7], in particular multiple sclerosis [8, 9] and acute measles encephalomyelitis [10, 11]. How viral infections can break tolerance and thereby lead to autoimmune conditions remains largely unknown. Possible mechanisms underlying a virus-induced autoreactive T-cell response include the presentation of crossreactive antigens (molecular mimicry) [12–14], the release of otherwise sequestered antigens from immunologically privileged sites [15, 16], the modification of the antigenicity of self antigens [17], and disruption of the regulation by suppressor T cells [18]. Furthermore, an infection can specifically activate self-reactive T cells by providing appropriate costimulation [19], *e.g.*, through the upregulation of costimulatory molecules on APC, or specifically by the release of cytokines or other molecular danger signals (bystander activation) [6, 20]. In addition, a viral infection may lead to the expression of nonphysiologic levels of self-peptides presented by major histocompatibility complex (MHC) molecules, either due to alterations in MHC class I or II expression [21, 22] as a result of the inflammatory response, or by modulating the peptide repertoire bound by MHC molecules [5, 23, 24].

To explore whether the latter mechanism may also play a role in the induction of MV associated autoimmune diseases we studied the possible shift in the MHC class I peptide display as a result of MV infection. For this, we performed a subtractive analysis of HLA-A*0201-associated peptides isolated from a human B-lymphoblastoid cell line (B-LCL) prior to and after MV infection, using sensitive mass spectrometric techniques. In addition to virus-derived peptides [25], we identified a non-viral peptide that was specifically induced by MV infection. This peptide was derived from a type I interferon inducible protein (IFI-6-16) of unknown function. Furthermore, we detected a virus-induced shift in the abundance of numerous self-peptides. From these, one abundant peptide, the expression of which was further upregulated following MV infection, was derived from

the β chain of a heat shock protein, Hsp90β. As a member of the Hsp90 family of well-conserved, cytosolic chaperone proteins, Hsp90 is one of the most abundant heat shock proteins in eukaryotic cells, even in the absence of stress stimuli [26]. We found that T cells capable of recognizing both self-peptides in the context of HLA-A*0201 are present in the human T-cell repertoire at birth, and autoreactivity against these peptides can be detected following MV infection.

MATERIAL AND METHODS

Virus, Cell Lines, and Infection

Plaque-purified MV (Edmonston B strain), grown in Vero cells, which were cultured in DMEM supplemented with antibiotics and 10% FCS, containing 10^7 tissue culture infectious $dosis_{50}/ml$, was used to infect the HLA-A*0201 homozygous Epstein-Barr virus (EBV) transformed B-LCL BSM, or the melanoma cell line MelJuSo at a multiplicity of infection of 1 in RPMI 1640 medium supplemented with antibiotics and 2% FCS. BSM and MelJuSo cells were cultured in RPMI 1640 medium supplemented with antibiotics and 10% FCS. The TAP deficient T2 cell line was adapted to grow in protein free hybridoma medium (PFHM) supplemented with antibiotics and 1% FCS.

Patients Studied

Cord blood, obtained from normal deliveries of newborns of 37 weeks or more of gestation, and blood samples of healthy donors were collected in The Netherlands. Blood samples from patients with measles were collected in the Gambia. All samples were collected according to the informed consent guidelines of the medical ethic committees from local hospitals. Cord blood mononuclear cells (CBMC) and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on fycoll-hypaque (Pharmacia Biotech, Uppsala, Sweden) and cryopreserved until used in experiments. Small aliquots of cells were used for DNA preparation and molecular HLA-typing.

Peptide Synthesis

The synthetic peptides SLMSWSAIL ($IFI-6-16_{74-82}$) and ILDKKVEKV ($Hsp90\beta_{570-578}$) were prepared by standard solid phase Fmoc chemistry using an ABIMED AMS 422 simultaneous multiple peptide synthesizer (ABIMED Analysen-Technik GmbH, Langenfeld, Germany).

Isolation and HPLC Fractionation and Mass Spectrometry

HLA-A*0201 molecules were immunoprecipitated from MV-infected and uninfected BSM cells as described previously [25] using the HLA-A*0201 specific monoclonal antibody BB7.2. Peptides were eluted and fractionated in four large fractions on a 2.1 mm × 10 cm reversed-phase high performance liquid chromatography (rpHPLC) C2/ C18 column (Pharmacia, SMART system) using an acetonitrile gradient (0%–60%) and 0.1% trifluoroacetic acid (TFA) in water (flow 100 μl/min).

Peptide fractions were analyzed using microcapillary rpHPLC electrospray ionisation mass spectrometry (μLC-ESI-MS) [27]. Briefly, ion traces in corresponding rpHPLC fractions of infected and uninfected cells were compared, mass by mass (abundant peptides present in both ion traces were used to assess small shifts in retention time). Peptides unique to or highly upregulated in the infected samples were sequenced by tandem MS analysis at optimized collision activated dissociation (CAD) energy. If product ion spectra contained insufficient sequence information, peptide samples were re-analyzed after on-column derivatization with N-hydroxysuccinimidyl-3-pyridylacetate (SPA), which helps to elucidate sequence information by promoting b-type ions and distinguishing between the isobaric amino acid residues K and Q [28]. Protein database searching was performed (http://www.mann.embl-heidelberg.de) to determine the source protein of the sequenced peptides. The sequence of the allocated peptides was confirmed using synthetic peptides.

For quantitation of the amount of a peptide in a sample, new peptide mixtures were generated, derived from approximately 10^9 cells that were spiked with known amounts of two synthetic peptide standards (Angiotensin III and Oxytocin; Sigma-Aldrich, St Louis, MO, USA) directly after peptide isolation to correct for sample loss during the subsequent processing of the samples. Quantitation of peptides in the mixture was performed on the summed intensity of the different charge states of a peptide ($[M+H]^+ + [M+2H]^{2+} + [M+3H]^{3+}$) at the expected retention time including possible oxidized forms, relative to the response obtained for the internal standards in full scan single ms μLC-ESI-MS analysis. Quantitation was based assuming equal specific responses (counts per pmol) and equal losses during sample processing for all peptides and the internal synthetic standards.

HLA-A*0201 Binding

The T2 peptide-binding assay was performed as described previously [25]. Briefly, T2 cells were incubated overnight with peptide at various concentrations of pep-

tide or in PBS alone. Peptide binding to the otherwise unstable HLA-A*0201 molecule will increase its expression at the cell surface, which was detected by the FITC-conjugated HLA*0201 specific monoclonal antibody (mAb) BB7.2 on a FACScan and analyzed using CellQuest software (Becton Dickinson, San Jose, CA, USA). Peptide binding was calculated as the fluorescence index (FI): FI = [(mean fluorescence in the presence of peptide) − (mean fluorescence without peptide)]/(mean fluorescence without peptide). In this assay, at a peptide concentration of 100 μM the FI is indicative of a low (0.5 < FI < 1.0), intermediate (1.0 < FI < 1.7) or high (FI > 1.7) affinity binding of the HLA-A*0201 molecule to the peptide [25].

Protein Expression and Localization

BSM cells were infected with MV for 24 hours, fixed with 2% paraformaldehyde, washed and incubated with 0.1-M glycine for 10 minutes, and permeabilized with 0.1% saponin/1% BSA in PBS. Immunostaining was performed with polyclonal rabbit-anti-IFI (RIVM, The Netherlands) and goat-anti-Hsp90β (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as primary antibodies, and donkey-anti-rabbit-IgG-FITC and donkey-anti-goat-IgG-FITC as secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Stained cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson).

The subcellular localisation of IFI-6-16 and Hsp90β was studied by confocal microscopy as described previously [29] using the antibodies described above, and propidium iodide (PI) (100μg/ml) for DNA counterstaining. FITC and PI fluorescence were pseudocolored in green and red respectively and high intense fluorescence (Channels 150–255) was pseudocolored as yellow in an overlay.

Tetrameric Complexes and FACS-Staining

MHC-peptide tetramers were generated essentially as described by Tan et al. [30]. In brief, HLA-A*0201 heavy chain containing a specific biotinylation site and β_2-microglobulin were expressed in large amounts in E. coli transformed with the appropriate plasmids, isolated by French press treatment, dissolved in 8 M of urea, and diluted into refolding buffer (400-mM L-arginine, 5 mM reduced glutathion, 0.5 mM oxidized glutathion, 2 mM EDTA, 100 mM Tris, pH 8) containing high concentrations of synthetic peptide. The generated monomeric, soluble HLA-A*0201-peptide complexes were biotinylated by the BirA enzyme (Avidity, Denver, CO, USA) followed by purification by gel filtration. Tetramer formation was performed by addition of PE-conjugated streptavidin (Sigma, St Louis, MO, USA), or APC conjugated streptavidin (Molecules Probes, Eugene, OR, USA) at a molar ratio of 4:1.

Tetramer staining of CBMC, PBMC or isolated CD8$^+$ T-cell fractions (positively selected with immunomagnetic beads [Miltenyi Biotech, Auburn, CA, USA] according to manufacturers instruction) was performed by incubating cells for 15 minutes at 37°C with the appropriate tetramer in FACS buffer (1% FCS in PBS) followed with an additional incubation with PerCP- or APC-conjugated anti-CD8 plus anti-CD3-FITC for 15 minutes at 37°C. Stained samples were subsequently washed with cold FACS buffer and approximately 30,000 events were measured in the lymphocyte gate combined with either a CD8$^+$ gate or a CD8$^+$/CD3$^+$ gate on a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson). Control samples for the tetramer staining were the CD8$^+$ enriched cellular fractions of PBMCs from HLA-mismatched donors.

IFNγ Enzyme-Linked Immunospot Assay

Peripheral blood mononuclear cells were pulsed with 10 μM of self-peptide or with control peptide for 1 hr before adding the cells to duplicate wells of polyvinylidene difluoride backed plates (MAIP S 45; Millipore, Bedford, MA, USA) coated with anti-IFN-γ antibody (Mabtech, Cincinnati, OH, USA). For PBMC of MV patients 1 × 10^4–1 × 10^5 cells were seeded per well, and for healthy control MV-immune donors 1 × 10^5–2.5 × 10^5 cells were seeded per well. Cells were incubated for 20–24 hours at 37°C, 5% CO$_2$. Plates were washed, developed with chromogenic alkaline phosphatase substrate after the two-step incubation with biotinylated anti-IFN-γ and streptavidin antibodies, respectively (Mabtech), and screened for IFNγ producing spot forming cells (SFC). Peripheral blood mononuclear cells obtained from Gambian patients in the acute phase were directly tested. For detection of epitope-specific memory responses in individuals after recovery, isolated PBMC from the same donors after recovery need to be stimulated first (A.J., unpublished observations). In the stimulated cultures, cells were grown for 10 days in complete RPMI 1640/10% FCS, after pulsing with 10 μM peptide, in the presence of 10% IL-2 (Lymphocult T; Biotest AG, Dreieich, Germany) and 5 ng/ml IL-7 (Genzyme, Cambridge, MA, USA). IL-2 was added at day 3 and day 7. At day 10 cells were pulsed with 10 μM peptide before adding them to Elispot plates and overnight incubation.

RESULTS

MV Infection Induces or Upregulates Abundant Expression of Two Self-Peptides Binding to HLA-A*0201

To study possible shifts in the display of peptides bound by HLA-A*0201 molecules as a result of MV infection,

HLA-A*0201-associated peptides were eluted from HLA-A*0201 molecules isolated from the homozygous B-LCL BSM prior to and 48-hours after MV infection. The comparison of the thousands of peptides present in both peptide mixtures revealed several individual peptide species that were only present in the post-infection peptide sample. Through collision activated dissociation (CAD) and tandem MS analysis of these MV-induced peptide species, amino acid sequences were obtained from a limited number of peptides, including three viral peptides [25]. Another MV-induced peptide, detected as its [M+H]$^+$ ion at m/z 1007.5 and sequenced as SL$_x$M-SWSAL$_x$L$_x$ (Figure 1A–E; L$_x$ stands for either L or I), did not match a MV protein sequence but showed full homology with amino acids 74-82 of the human type I IFN inducible protein 6-16 (IFI-6-16$_{74-82}$, i.e., SLMSW-SAIL). The identification of the biologic peptide ion at m/z 1007.5 as IFI-6-16$_{74-82}$ was confirmed after comparison with synthetic SLMSWSAIL, which had the same μLC retention time and CAD spectra (data not shown).

During the subtractional analysis of HLA-A*0201 eluted peptides prior to and after MV infection we also observed several peptide species of which the expression was enhanced or reduced by MV infection of the cell. Notably, MV infection did not alter the expression of MHC class I molecules itself on infected BSM cells (data not shown). One naturally processed non-viral peptide that was at least five times enhanced in the infected peptide mixture, was detected at its [M+H]$^+$ ion at m/z 1071.8 and was sequenced as L$_x$L$_x$D[K/Q] [K/Q]VE[K/Q]V (Figure 1F–J). This sequence was homologous to amino acids 570-578 from the β chain of human Hsp90 (Hsp90β$_{570-578}$, i.e., ILDKKVEKV), and their identity was confirmed using synthetic ILDKKVEKV.

In subsequent repetitive peptide isolation experiments, the presence and abundance of IFI-6-16$_{74-82}$ and Hsp90β$_{570-578}$ in HLA-A*0201 on MV-infected and uninfected cells was further studied. Consistently, no IFI-6-16$_{74-82}$ was detected in peptide mixtures obtained from uninfected cells, while the amount of IFI-6-16$_{74-82}$ recovered per aliquot of 10^9 infected cells varied between 0.9 pmol and 5 pmol, corresponding to an average density of 500 and 3000 copies per cell respectively. Herewith this epitope ranks as a relatively abundant HLA class I ligand [31, 32]. The amount of Hsp90β$_{570-578}$ recovered from uninfected cells varied between 7 pmol and 24 pmol per 10^9 cells, corresponding to 4000 and 14,000 copies per cell, respectively. These data indicate that the basal level of expression of Hsp90β$_{570-578}$ is already high and variable. However, the amount of this peptide recovered from infected cells varied between 7 pmol and 70 pmol per 10^9 cells, corresponding to 4000 and 40,000 copies per infected cell, respectively, quali-
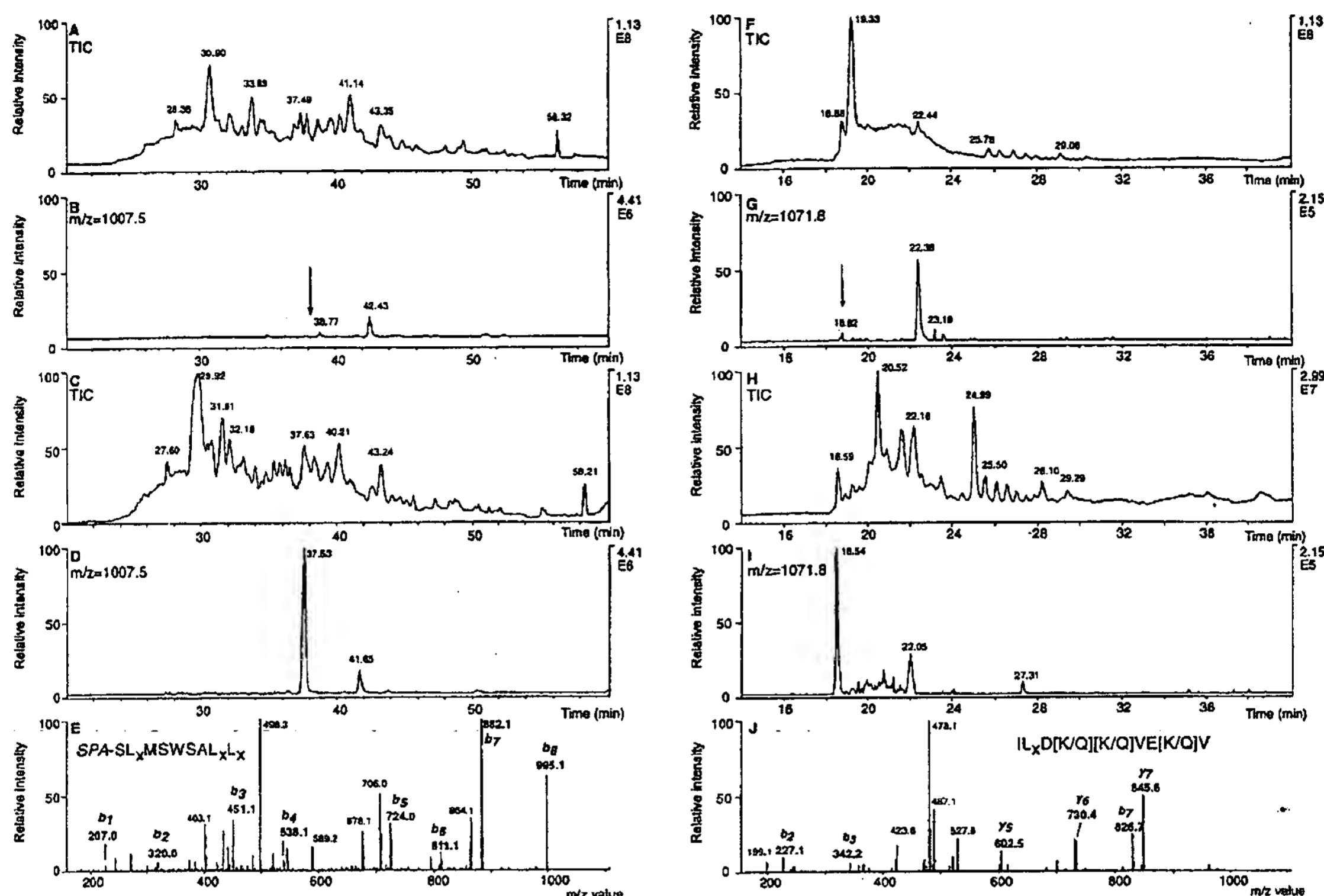
FIGURE 1 Detection and identification of two naturally processed self-peptides in HLA-A*0201 that are induced or upregulated by measles virus (MV) infection. Peptides were isolated from BSM cells prior to and after MV infection and fractionated into four large fractions by rpHPLC as described in material and methods. Shown are total (A, C, F, and H) and extracted (B, D, G, and I) ion currents recorded in rpHPLC-fraction 2 (F–I) and 4 (A–D) from MV-infected (C, D, H, and I) and uninfected (A, B, F, and G) cells, and CAD analyses of SPA-derivatized ions (E, J). A peptide species at m/z 1007.5

was present in HPLC-fraction 4 from the MV infected cells (D) and absent in the corresponding HPLC-fraction of the uninfected cells (B). CAD analysis of this peptide after on-column SPA-derivatization was determined to be $SL_xMSWSAL_xL_x$ (where L_x stands for either L or I)(E). A peptide species at m/z 1071.8 was at least five times more abundant in HPLC-fraction 2 of the infected cells (I) than in the corresponding HPLC-fraction of the uninfected cells (G). The sequence of this peptide was determined by CAD analysis to be $IL_x[K/Q]$ [K/Q]VE[K/Q]V (J).

fying the expression of this self-peptide on MV-infected cells as very highly abundant [31, 32]. Even though in none of these experiments the initial fivefold enhancement in the expression of $Hsp90\beta_{570-578}$ following MV infection was reached, the general trend of a MV-induced increase in its expression was confirmed.

To determine the affinity of the HLA-A*0201 molecule for $IFI-6-16_{74-82}$ and $Hsp90\beta_{570-57}$ we used an assay based on the capacity of a peptide to stabilize the cell surface expression of empty unstable HLA-A*0201 molecules on the TAP-deficient T2 cells. We found that even though the affinity of HLA-A*0201 for both self-peptides was lower than for other CTL epitopes tested (Table 1), $IFI-6-16_{74-82}$ and $Hsp90\beta_{570-578}$ are still bound by this restriction element with a relatively high affinity (FI > 1.7).

Expression and Subcellular Localization of IFI-6-16 and Hsp90β Before and After MV Infection

The observed upregulation of two self-peptides in HLA-A*0201 on MV-infected cells suggests that the expression of their parental proteins becomes modulated by MV infection. To study this we first assessed the intracellular expression of the IFI-6-16 and the Hsp90β proteins in infected and uninfected BSM cells by flow cytometry. IFI-6-16-specific immunofluorescence was readily detected in uninfected BSM cells, and was enhanced as a result of viral infection (Figure 2A), indicating that in contrast to its peptide, expression of IFI-6-16 protein is not restricted to MV-infected cells. Intracellular staining for HSP90β was found in untreated BSM

TABLE 1 Peptide binding affinity to HLA-A*0201

Peptide	Sequence	Affinity to HLA-A*0201 (FI at 100 μM)
IFI-6-16$_{74-82}$	SLMSWSAIL	2.3
Hsp90β$_{570-578}$	ILDKKVEKV	3.1
HPV 16 E7$_{86-93}$	TLGIVCPI	5.7
Flu-M$_{57-65}$	GILGFVFTL	4.6
MV-C$_{84-92}$(L$_{85}$P)	KPWESPQEI	0.1

Abbreviations: FI = fluorescence index; Flu-M$_{57-65}$ = Influenza matrix protein$_{57-65}$; Hsp90β = heat shock protein 90β chain; HLA = human leukocyte antigen; HPV 16 E7$_{86-93}$ = human papillomavirus 16 E7 protein$_{86-93}$; IFI-6-16 = type I interferon inducible protein; MV = measles virus.

cells, and was upregulated after MV infection (Figure 2B).

We next investigated whether MV infection induced a shift in the subcellular distribution of IFI-6-16 and Hsp90β by studying their compartmentalization with confocal microscopy in MelJuso cells, which can be readily infected with MV [29]. In uninfected cells IFI-6-16 localizes to the nucleus and the cytoplasm, where it is present in tubular structures while absent from the perinuclear region, a pattern showing a marked resemblance with the structure of the endoplasmic reticulum (Figure 3A). MV infection induced small changes in the subcellular distribution of IFI-6-16 towards a more granular like pattern in both nuclei and cytoplasm (arrowhead in Figure 3B), together with a minor increase of the fluorescence intensity in the nucleus (Figures 3A and 3B, see also inlets). In uninfected cells Hsp90β was predominantly detected as a fine granular, sometimes diffuse fluorescence pattern in the cytoplasm (Figure 3C). A clear enhanced cytoplasmic fluorescence together with a decreased localization in the nucleus was observed in MV-infected cells (Figures 3C and 3D, see also inlets, and legends for different gain settings for Figures 3C and 3D). Taken together, the induced presentation of an IFI-6-16-derived epitope in HLA-A*0201 molecules on MV-infected cells was only accompanied by subtle changes in IFI-6-16 protein expression after infection. In contrast, the upregulation of Hsp90β$_{570-578}$ in HLA-A*0201 as a result of MV infection paralleled an overall increased expression of the parental protein Hsp90β, and a further accumulation in the cytosol.

T Cells Recognizing IFI-6-16$_{74-82}$ and Hsp90β$_{570-578}$ are Present but Functionally Silent in the Naïve and Adult Human T-Cell Repertoire

To determine whether CD8$^+$ T cells capable of recognizing IFI-6-16$_{74-82}$ and Hsp90β$_{570-578}$ in the context of HLA-A*0201 molecules are present in the human T-cell repertoire, we constructed tetrameric complexes of HLA-A*0201 molecules containing these self-peptides. First, we studied the presence of CD8$^+$ T cells that were

Fluorescence intensity →
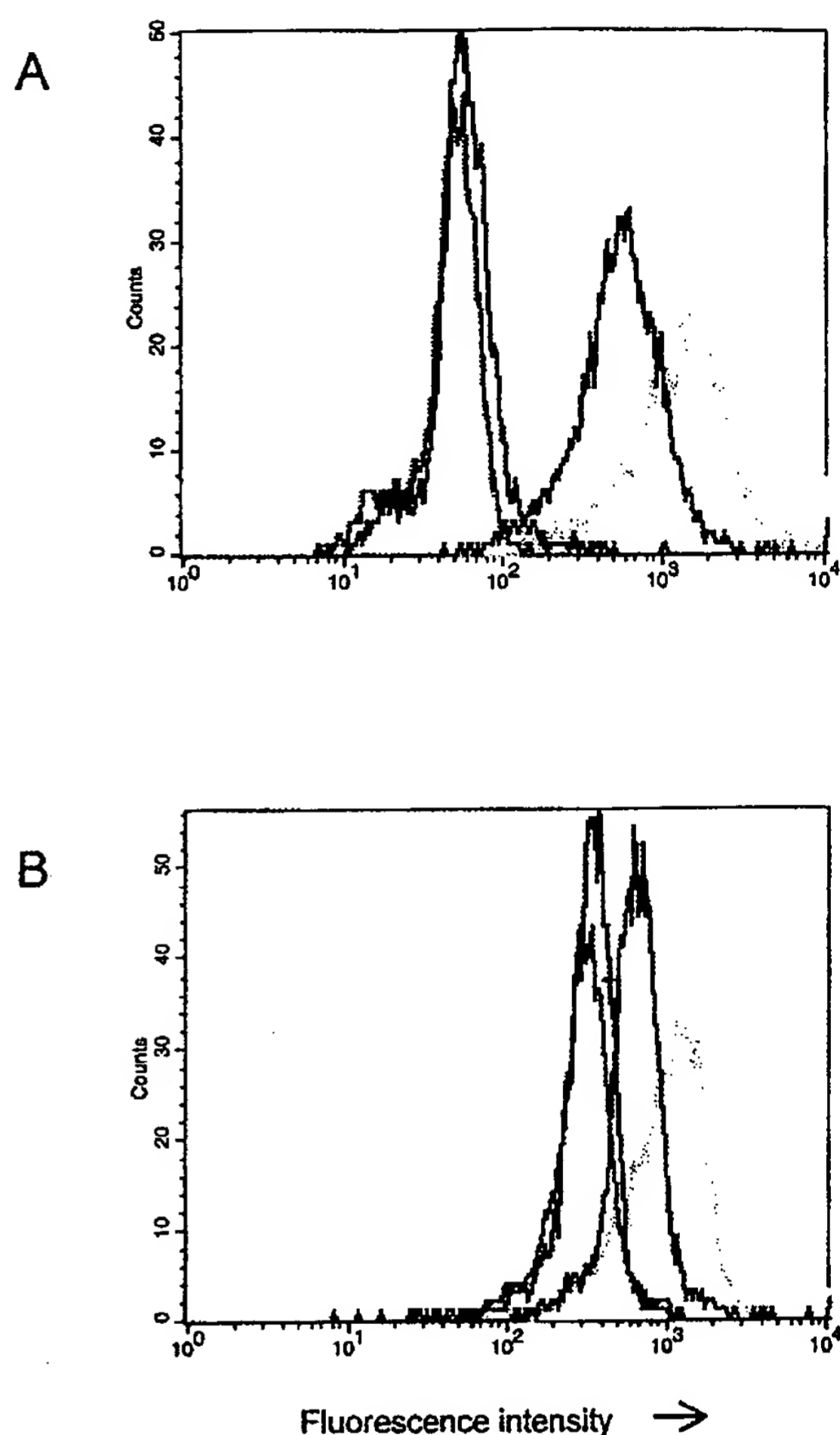
FIGURE 2 Modulation of intracellular expression of IFI-6-16 and Hsp90β proteins by measles virus (MV) infection. Immunofluorescent staining for IFI-6-16 (A) and Hsp90β (B) was assessed in the presence of specific antibodies and FITC labeled conjugate (yellow, green), or of FITC labeled conjugate only (pink, blue), in MV infected (yellow, pink) or uninfected (green, blue) BSM cells.

able to bind to the HLA-A*0201/IFI-6-16$_{74-82}$ and the HLA-A*0201/Hsp90β$_{570-578}$ tetrameric complexes in naïve CBMC enriched for CD8$^+$ cells. In all HLA-A*0201 positive cord blood donors (n = 3), HLA-A*0201/IFI-6-16$_{74-82}$ positive CD8$^+$ T cells could be detected with frequencies ranging from 0.2%–0.4% of the total CD8$^+$ T-cell population, whereas for HLA-A*0201/Hsp90β$_{570-578}$ frequencies ranged from less then 0.1% to 1.1% of all CD8$^+$ T cells (Figures 4A–4C). These findings suggest that measurable numbers of IFI-6-16$_{74-82}$ and in some cases also Hsp90β$_{570-578}$ specific
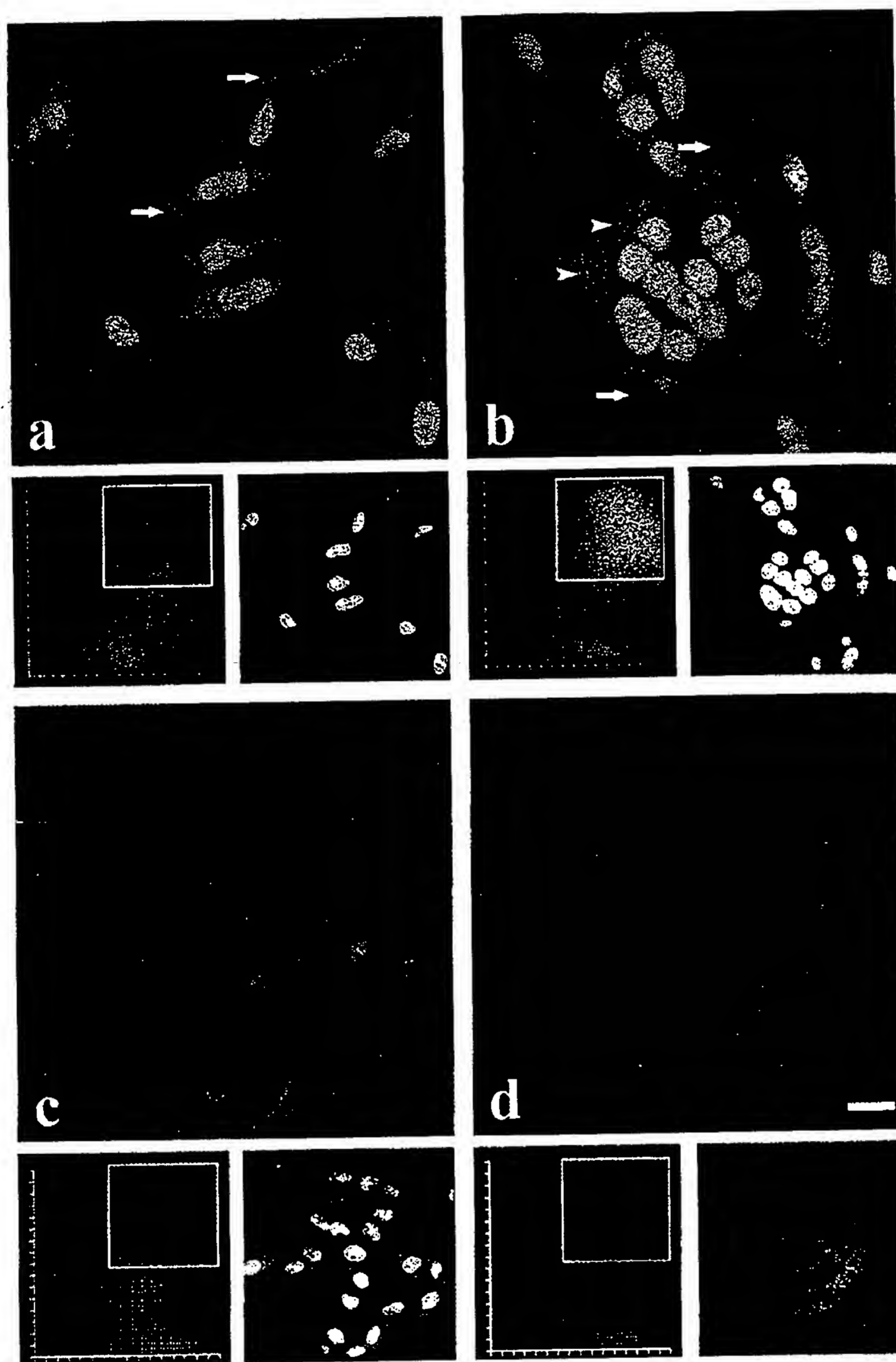
FIGURE 3 Minor and major shifts in the subcellular localization of IFI-6-16 and Hsp90β, respectively, following measles virus (MV) infection. Uninfected (A and C) and MV-infected (B and D) MelJuso cells were immunostained for intracellular IFI-6-16 (A and B) or Hsp90β (C and D). Immunofluorescence for IFI-6-16 and Hsp90β is illustrated as green and DNA fluorescence as red. IFI-6-16 is present in the cytoplasm as tubular structures (arrows) and absent in the perinuclear region, a more granular-like staining becomes evident after MV infection (arrowhead). A high fluorescence intensity for IFI-6-16 is present in the nucleus of non-infected and MV-infected cells (inlets A and B where nuclear immunolocalization was selected [box] and pseudocolored). A granular, sometimes diffuse immunofluorescence is seen for Hsp90β in non-infected and MV-infected cells (C, D). MV infection enhances the Hsp90β-fluorescence intensity (note: gain-settings of the confocal microscope were adjusted in D, reducing total Hsp90β specific fluorescence, compare uninfected mononuclear cell in control sample (C, middle) with uninfected mononuclear cells in infected sample (D, top-left), and reduces the presence of Hsp90β in the nucleus (inlets C and D). Bar represents 10 μm.
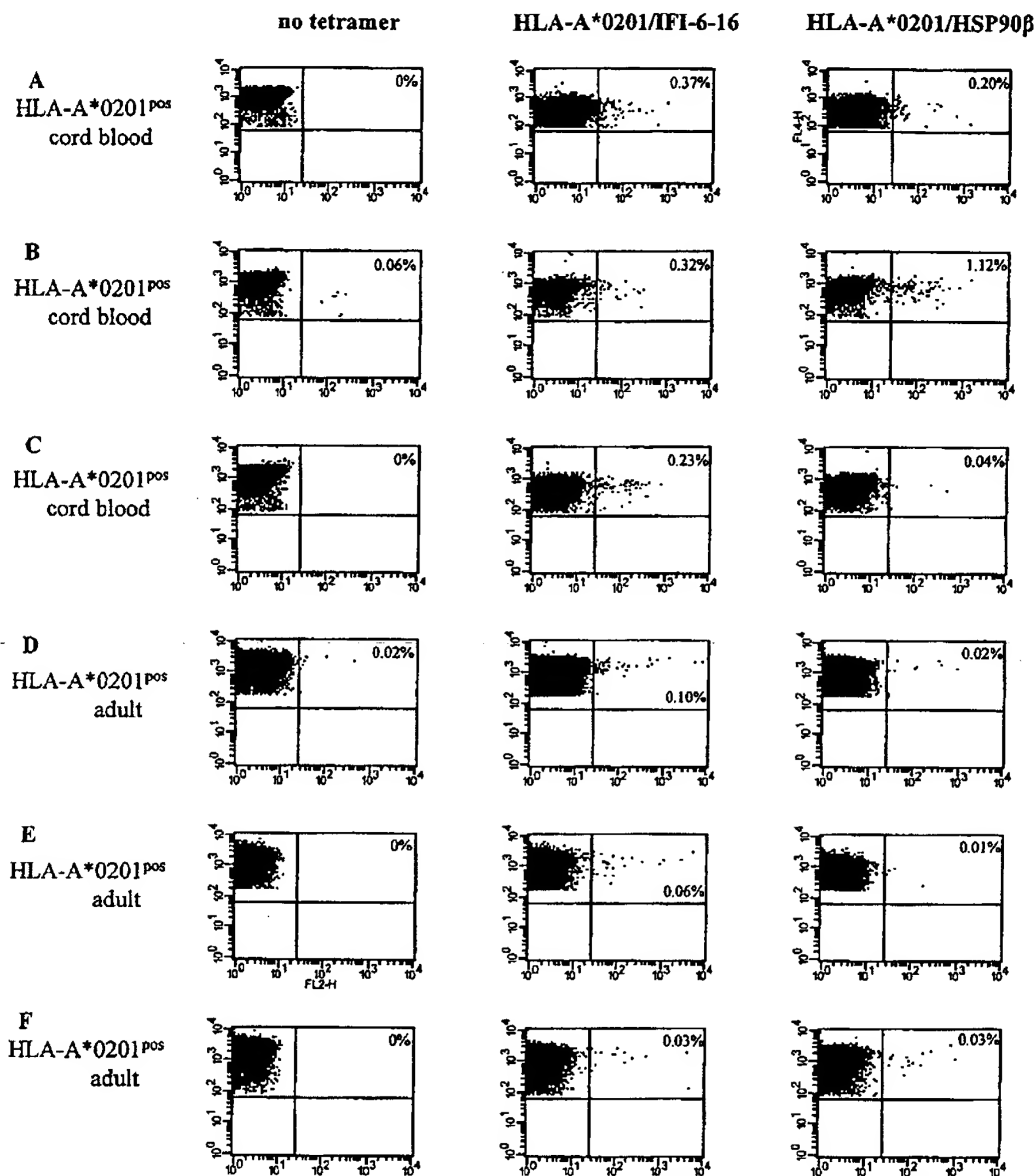
FIGURE 4 $IFI-6-16_{74-82}$ and $HSP90\beta_{570-578}$ binding $CD8^+$ T cells are present in peripheral blood mononuclear cells (PBMC) from HLA-A*0201-positive healthy individuals. Illustrated are $CD8^+$ enriched cord blood mononuclear cells (CBMC) from three HLA-A*0201 positive cord blood donors (A–C) and $CD8^+$ enriched PBMC from three MV immune HLA-A*0201 positive adults (D–F) stained with CD8 and/or CD3 without tetramer, with $HLA-A*0201/IFI-6-16_{74-82}$ tetramer or $HLA-A*0201/Hsp90\beta_{570-578}$ tetramer as indicated. Illustrated are dot plots of $CD8^+$ cells (D–F) or $CD8^+/CD3^+$ cells (A–C) in the lymphgate with tetramer staining on the x-axis and CD8 staining on the y-axis. The percentages of tetramer positive $CD8^+$ cells are indicated in the upper right quadrant of the dot plot. Staining patterns observed in HLA-A*0201 positive individuals were always brighter that those obtained in HLA-A*0201 negative controls (not shown) and therefore considered as genuinely epitope-specific.

CTL precursors are present in the human T-cell repertoire in HLA-A*0201 positive individuals at birth.

Next, we assessed the levels of $HLA-A*0201/IFI-6-16_{74-82}$-binding and $HLA-A*0201/Hsp90\beta_{570-578}$-binding $CD8^+$ T cells in peripheral blood from HLA-A*0201 positive adults with a history of MV infection or vaccination in childhood ($n = 8$). With the exception of one highly reactive donor (not shown), in all donors (illustrated for three in Figures 4D–4F) frequencies of tetramer staining $CD8^+$ T cells were consistently low ($<0.1\%$ $CD8^+$ T cells). Stimulation of PBMC from these adult donors with the $IFI-6-16_{74-82}$ or $Hsp90\beta_{570-578}$ peptide for approximately 24 hours did not result in the detection of peptide-specific IFN-γ producing SFC in

TABLE 2 IFN-γ producing cells upon peptide stimulation in MV patients

MV patient	Peptide	Acute phase	Recovery
M300	IFI-6-16$_{74-82}$	3 (0)*	28 (23)
	Hsp90β$_{570-578}$	16 (0)‡	25 (23)
	MV-H$_{30-38}$	16 (0)‡	45 (23)†
M571	IFI-6-16$_{74-82}$	13 (3)†	32 (17)†
	Hsp90β$_{570-578}$	13 (3)†	13 (17)
	MV-H$_{30-38}$	14 (3)†	27 (17)†

* SFC found in the presence of peptide, between bracket background SFC (found in the absence of peptide).

$^\dagger p < 0.05$

$^\ddagger p < 0.01$

Number of cells seeded per well were 3 × 10^4 (M300, acute phase), 1 × 10^5 (M300, recovery), 5 × 10^4 (M571, acute phase) and 1 × 10^4 (M571, recovery).

Abbreviations: Hsp90β = heat shock protein 90β chain; IFI-6-16 = type 1 interferon inducible protein; IFN = interferon; MV = measles virus; SFC = spot forming cell.

ELISPOT (detection limit <2 SFC/10^5 PBMC, data not shown). These data suggest that even though basal levels are generally lower than those found in naïve cord blood, potentially autoreactive T cells recognizing IFI-6-16$_{74-82}$ or Hsp90β$_{570-578}$ in the context of HLA-A*0201 are present but functionally unresponsive in the adult human MV-immune CD8$^+$ T-cell repertoire.

IFN-γ Producing Effector Cells Recognizing IFI-6-16$_{74-82}$ and Hsp90β$_{570-578}$ are Present in MV Patients

To assess whether the altered expression of self-peptides in MHC class I could lead to the functional activation of autoreactive T cells recognizing the self-ligand *in vivo*, PBMC from two HLA-A*0201 positive MV patients were tested for their capacity to produce IFN-γ upon stimulation with the IFI-6-16$_{74-82}$ and Hsp90β$_{570-578}$ peptides in ELISPOT. We found that IFN-γ producing SFC specific for both peptides can be present in PBMC from acutely infected patients up to a level of 20 and 53 SFC per 10^5 PBMC for IFI-6-16$_{74-82}$ and Hsp90β$_{570-578}$, respectively (Table 2). In the recovery samples of these two MV patients (5–7 weeks after the rash), the IFI-6-16$_{74-82}$-specific IFN-γ response was maintained, but no peptide-specific memory response against the Hsp90β$_{570-578}$ peptide could be detected in this assay. As a control, peptide-specific responses against several other peptides were maintained in both patients during this period (shown for MV-H$_{30-38}$; Table 2). To study if MV infection also induced the expansion of the small numbers of CD8$^+$ T cells binding to the HLA-A*0201/IFI-6-16$_{74-82}$ and the HLA-A*0201/Hsp90β$_{570-578}$ tetrameric complexes, PBMCs obtained from five other HLA-A*0201 positive MV patients during acute infection and upon recovery were stained with these tetramers. In all patients the pattern and

frequencies of CD8$^+$ tetramer binding cells were similar to those of uninfected healthy adults (data not shown). Thus, whereas natural MV infection does not induce an apparent expansion of the IFI-6-16$_{74-82}$ or Hsp90β$_{570-578}$ specific CD8$^+$ T cells in the peripheral blood, it does induce the functional activation of these cells.

DISCUSSION

In this study we provide direct evidence for a mechanism underlying the virally induced activation of autoreactive T cells. We found that MV infection causes a shift in the MHC class I peptide display on human B-lymphocytes, a natural target cell of this virus [33]. In addition to the appearance of MHC class I presentation of viral peptides [25], MV infection also altered the presentation of peptides derived from self-proteins. Here, we describe the identification of two self-peptides in HLA-A*0201, IFI-6-16$_{74-82}$ and Hsp90β$_{570-578}$, the expression of which was either induced or upregulated to abundant levels following MV infection. Their parental proteins, IFI-6-16 and the β chain of Hsp90, respectively, were so far not implicated in MV pathogenesis.

IFI-6-16, a putative 12 kD transmembrane protein was first identified from mRNA that was highly induced in cells treated with type I IFN [34, 35], and poorly, if at all, in response to IFN-γ [35, 36]. The here reported subcellular localization of IFI-6-16 in untreated cells suggests a function in the endoplasmic reticulum and the nucleus. Notably, the IFI-6-16$_{74-82}$ epitope was undetectable in uninfected cells, and became only apparent after MV infection. The minor increase of IFI-6-16 protein expression after MV infection, known to induce the production of type I IFNs [37–39], as well as the observed shift towards cytosolic localization of this protein, could point at an altered intracellular processing route of IFI-6-16 leading to the de novo generation of IFI-6-16$_{74-82}$/HLA-A*0201 complexes in MV-infected cells.

The Hsp90 αβ heterodimer is an abundant cytosolic chaperone protein essential for the viability of eukaryotic cells, and upregulated under stress conditions such as heat shock and inflammation [26]. As could be expected for a subunit of a protein interacting with the proteasome [40, 41], we readily found cytoplasmic staining for Hsp90β in uninfected cells. Also we found basal, yet variable amounts of the HLA-A*0201 presented Hsp90β$_{570-578}$ epitope in uninfected cells, indicating that the Hsp90β protein normally intersects the MHC class I processing pathway. Since the Hsp90αβ protein is a component of the stress response, variation of the basal Hsp90β$_{570-578}$ epitope level could well be attributed to subtle differences in stress conditions between individual semi-large scale nonautomated cell cultures (>10^9 cells) needed for peptide isolation. Apparently, the additional

stress of a viral infection further enhances the formation of $Hsp90\beta_{570-578}$/HLA-A*0201 complexes on MV-infected cells. The $Hsp90\beta_{570-578}$ enhancement is paralleled by an increased expression and further cytosolic accumulation of the parental protein, indicating a rise in the amount of $Hsp90\beta$ available for proteasomal degradation and further MHC class I processing after MV infection. Using the DNA microarray technology, others have recently included several genes of the IFN and stress response systems to be upregulated by MV infection [37]. Our study directly highlights the consequences hereof for MHC-peptide presentation.

Owing to their altered infection-related generation and their relatively high-affine binding to HLA-A*0201, the $IFI-6-16_{74-82}$ or $Hsp90\beta_{570-578}$ epitopes become potential target epitopes for autoreactive $CD8^+$ T cells. T cells binding to either $IFI-6-16_{74-82}$ or $Hsp90\beta_{570-578}$ presented in the context of HLA-A*0201 were indeed detectable in the human T-cell repertoire, apparently having escaped from negative selection during thymic residence. Since we found that both parental proteins are expressed under normal conditions in cells of lymphoid and melanocyte origin and participate in the host's stress response, we assume that IFI-6-16 and $Hsp90\beta$ do not have a tissue specific expression pattern but are also present in the thymus. Lack of tolerance induction may therefore point at crypticity of their respective epitopes in the thymus, being expressed either too poorly or not at all, to induce clonal deletion [42]. Processing events unique to the thymus may underlie such crypticity [43].

We found that while considerable in cord blood, basal levels of $IFI-6-16_{74-82}$-HLA-A*0201 tetramer or $Hsp90\beta_{570-578}$-HLA-A*0201 tetramer binding $CD8^+$ T cells had declined to almost undetectable levels in healthy MV-immune adults. Downregulation of T-cell receptors (TCR) expressed by potentially autoreactive T cells is one of the mechanisms proposed for the induction of peripheral tolerance [44, 45], and thus could explain this decline during life. Notably, the low numbers of tetramer-binding $CD8^+$ T cells present in MV immune adults were functionally inert in ELISPOT, but could be expanded in the presence of specific peptide and cytokines (C. Herberts, unpublished results), indicating that IFI-6-16_{74-82} and $Hsp90\beta_{570-578}$ specific cells are silently present in the periphery, yet have the capacity to proliferate.

Autoreactive T cells in the periphery will only become harmful when activated. In two HLA-A*0201 positive MV patients we found preliminary evidence for such activation of $IFI-6-16_{74-82}$ and $Hsp90\beta_{570-578}$ specific $CD8^+$ T cells. Self-peptide specific IFN-γ responses were detected in these patients in ELISPOT (Table 2), but were not correlated with an increase in the number of self-tetramer-staining $CD8^+$ T cells ex vivo. Although

these observations should be extended to more patients and controls, they do indicate that natural MV infection may lead to the functional activation of $IFI-6-16_{74-82}$- and $Hsp90\beta_{570-578}$-specific effector cells, but without measurable expansion. Thus, altered MHC self-peptide presentation, probably combined with molecular danger signals [20], can break tolerance of autoreactive T cells during viral infection.

The role of activated autoreactive $CD8^+$ T cells during viral infections is currently unknown. They could help clear the virus by eliminating infected cells. Once differentiated into memory T cells, they could also assist in the accelerated clearance of subsequent infections with the same or other infectious agents, provided these induce the same epitopes to be enhanced [46, 47]. In this context, autoreactive T cells specific for the self protein Hsp65 have been proposed to be play an immunoregulatory role at sites of inflammation and autoimmune tissue destruction [48].

On the other hand, virally activated autoreactive T cells may also be immunopathogenic by initiating autoimmunity [49]. In this respect, activated autoreactive T cells responding against virus-enhanced MHC class I self-ligands (e.g., $Hsp90\beta_{570-578}$) could potentially be more harmful than those against virus-induced self-peptides of which presentation is restricted to infected cells only (e.g., $IFI-6-16_{74-82}$), since the former may also attack non-infected tissue and may continue to do so even after recovery from infection. Notably, we found that IFN-γ responses against $IFI-6-16_{74-82}$, but not $Hsp90\beta_{570-578}$, could still be detected upon recovery from MV infection. Whether this indicates that self-reactivity against ubiquitous self-epitopes is more tightly controlled than that against peptides only emerging during infection is presently unknown.

Measles virus infection has been associated with several autoimmune diseases including multiple sclerosis [7]. Preliminary findings suggest the presence of moderately elevated numbers of $CD8^+$ T cells binding to the $HLA-A*0201/IFI-6-16_{74-82}$ or $HLA-A*0201/Hsp90\beta_{570-578}$ tetramers in individuals suffering from multiple sclerosis (C. Herberts, unpublished observations). Further functional characterization of these potentially autoreactive T cells is needed to gain insight into their possible role, either pathologic or protective, in (the onset of) autoimmunity.

Notably, infection with Epstein-Barr virus, which is also associated with multiple sclerosis, was found to induce MHC presentation of a peptide derived from the small heat shock protein αB-crystallin, a candidate autoantigen in multiple sclerosis [24]. Furthermore, di Marzo Veronese et al. [5] have described the induction of HLA-A2 restricted self-peptides derived from the cytoskeleton protein vinculin following chronic infection of

B-LCL cells with human immunodeficiency virus-1 (HIV-1). Similar to our findings, effector cells recognizing (one of) these peptides were readily detectable in a number of HLA-A2-positive HIV-1 infected individuals. Collectively, these data indicate that both acute and chronic viral infections can lead to the upregulation or induction of self-peptides in MHC molecules, and can be associated with the (temporary) activation of autoreactive effector cells recognizing these peptides. More insight into the expression and immunogenicity of self-epitopes associated with viral infections and their functionality, be it regulatory or pathogenic, may lead towards the development of better immunotherapeutic intervention strategies.

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